

Activation tagging of the *LEAFY PETIOLE* gene affects leaf petiole development in *Arabidopsis thaliana*

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SUMMARY

In a screen for leaf developmental mutants we have isolated an activator T-DNA-tagged mutant that produces leaves without a petiole. In addition to that leafy petiole phenotype this *lettuce* (*let*) mutant shows aberrant inflorescence branching and silique shape. The *LEAFY PETIOLE* (*LEP*) gene is located close to the right border of the T-DNA insert linked with these dominant phenotypes and encodes a protein with a domain with similarity to the DNA binding domain of members of the AP2/EREBP family of transcription factors. Introduction of the activation-tagged *LEP* gene in wild-type plants conferred

all the phenotypic aberrations mentioned above. The leafy petiole phenotype consists of a conversion of the proximal part of the leaf from petiole into leaf blade, which means that leaf development in *let* is disturbed along the proximodistal axis. Therefore, *LEP* is involved in either cell division activity in the marginal meristem or patterning along the proximodistal axis.

Key words: *LEAFY PETIOLE*, *lettuce*, Marginal meristem, Petiole development, Leaf blade, *Arabidopsis thaliana*

INTRODUCTION

The development of dicotyledonous leaves is a complicated process involving regulated division and expansion of cells (Tsukaya et al., 1994; Tsukaya, 1995), resulting in the patterning of a dorsoventral and proximodistal axis of asymmetry in the leaf primordium (Van Lijsebettens and Clarke, 1998; Poethig, 1997). The characterisation of the *phantastica* (*phan*) mutant of snapdragon (Waites and Hudson, 1995; Waites et al., 1998) and the *phabulosa* mutant of *Arabidopsis thaliana* (McConnell and Barton, 1998) showed that the establishment of dorsoventrality in the leaf primordium is an essential step for the initiation of marginal meristem activity leading to leaf blade formation. Although PHAN is necessary for the establishment of dorsoventrality, PHAN expression was observed throughout the whole leaf primordium. Therefore, it was proposed that PHAN interacts with other genes that show spatially restricted expression patterns or that PHAN activity is inhibited by a factor exhibiting specific expression in the ventral domain (Waites et al., 1998). Other genes that have been shown to be involved in the specification of dorsoventrality are members of the *YABBY* family of transcription factors specifying ventral cell fate and *PINHEAD/ZWILLE* (*PNH*) specifying dorsal cell fate (Bowman, 2000 and references therein). Interestingly, both the *YABBY* genes and *PNH* first show expression throughout the very young leaf primordium before their expression becomes restricted to the ventral or dorsal domain, respectively.

Therefore, it remains to be determined which genes are primarily responsible for the establishment of dorsoventrality.

In contrast to the number of mutants isolated affecting dorsoventral patterning only a few mutants have been isolated that are disturbed in the other patterning processes taking place in the leaf primordium. In the *lam-1* mutant of *Nicotiana sylvestris* (McHale, 1992, 1993) the blade initiation site is established normally, but subsequent cell division is disturbed (McHale and Marcotrigiano, 1998). *LAM* was shown to act after the establishment of dorsoventrality in the leaf primordium induced by *PHAN* and probably encodes a non-cell-autonomous dorsalising factor necessary for maintaining dorsoventrality in the developing leaf blade (McHale and Marcotrigiano, 1998). The *Arabidopsis arrested development3* (*add3*) mutant (Pickett et al., 1996) displays defects in the proper formation of a leaf blade and *ADD3* could be a gene with similar function to *LAM*.

To come to a better understanding of leaf development, we generated a pool of *Arabidopsis* lines harbouring an activator T-DNA construct (van der Graaff and Hooykaas, 1996; van der Graaff et al., 1996) that was screened for leaf developmental mutants. Here, we describe the phenotypic and genetic analysis of the dominant T-DNA-tagged *lettuce* (*let*) mutant, which exhibited a novel type of defect in leaf development. The mutant leaves lack a petiole and consequently, the leaves consist of a large leaf blade. This leafy petiole phenotype is the result of the conversion of petiole into leaf blade. We show that this altered leaf development is caused by activation tagging of

LEP, a gene that codes for a protein with similarity to the family of AP2/EREBP like transcription factors.

MATERIALS AND METHODS

Growth conditions and plant transformations

Seeds of the transgenic lines and wild-type control (all *Arabidopsis thaliana* ecotype C24) were either surface sterilised and grown in tissue culture on 1/2MS10 medium (van der Graaff and Hooykaas, 1996) or in soil under a 16/8 hours light/dark regime at 21°C. The pool of 550 transgenic lines harbouring the activator construct pSDM1550 was generated using a leaf transformation protocol as described before (van der Graaff and Hooykaas, 1998). The 35SDE-*LEP-mac12.6* and 35SDE-*LEP* constructs (Fig. 1B) were transformed to *Arabidopsis* using the root transformation protocol described by Vergunst et al. (1998) and the DE-*LEP* (Fig. 1B) and cDNA_{oe} constructs were transformed using the floral dip method (<http://www.cropsci.uiuc.edu/~a-bent/protocol.html>). The cDNA_{oe} construct was obtained by cloning the PCR-amplified *LEP* coding region (pLEP_{codF} and pLEP_{codR} primers) in the 35S overexpression cassette of pART7 (Gleave, 1992).

Genetic analysis of the *let* mutant

The original *let* mutant was isolated as a tetraploid transgenic line harbouring two independently segregating T-DNA loci. This mutant line was backcrossed two consecutive times with wild-type plants to obtain diploid, single locus lines. The (sub)lines resulting from these crosses all segregated for a single T-DNA locus and were diploid. The lines segregating for the dominant leafy petiole phenotype were used for linkage analysis. All seedlings exhibiting the leafy petiole phenotype in the progeny of these lines were hygromycin resistant (>200 seedlings) and all these lines harboured the same T-DNA insert in Southern blot analysis. Therefore, the leafy petiole phenotype was closely linked with this T-DNA insert. The isolation of the T-DNA insert with flanking plant DNA (Fig. 1A) and subsequent sequence analysis was performed as described by van der Graaff and Hooykaas (1996).

Primers

The following primers were used in this study (Fig. 1A). RTL1F: (41283) 5'-TACTCAGACATGCCTCCT-3', RTL1R: (41529) 5'-AGCTCATTACTACTGTCA-3', RTL2F: (42337) 5'-GATAAATGGGCAGAGCGT-3', RTL2R: (42733) 5'-CAATAGGACTGAGAAAA-GGT-3', GapCF 5'-AGCTCGTCGCTGTCAACG-3', GapCR: 5'-GACAGCCTTGGCAGCTCCT-3', 35SleaderF: 5'-TTCTTTCA-AATACTTCCAC-3', pLETF: (40363) 5'-GTATCTAAGGTAACA-TGCT-3', pLETR: (40804) 5'-GTGGCCCATGATATATCA-3', LET_{codF}: (41052) 5'-TTACCATGGGAAAGATGAACACAACA-3', LET_{codR}: (41690) 5'-AGACTGCAGATTAGGAGCCAAAGTA-3', LEP5'race: (41607) 5'-GACGAGTAGTCGTCACCGGTCCAG-3', LEP3'race: (41294) 5'-TGCCTCCTTCCTCATCCGTCACC-3' and PROM5'race: (40980) 5'-GAAGGAAGAGAGGGAATGTGTGT-TGG-3'. The numbers in brackets indicate the position of the primers on the BAC clone MAC12.

RNA isolation and analysis of gene expression

Total RNA was isolated from 2-week old plants grown in liquid culture (1/2MS10 medium) according to van Slogteren et al. (1983), unless otherwise stated. Northern analysis was performed as described in Memelink et al. (1994). Poly(A)⁺ RNA was isolated using the Oligotex mRNA kit (Qiagen). Transcripts were detected using [α -³²P]dCTP random labelled probes. To test for equal loading, blots were stripped and hybridised with a probe spanning the coding region of the *Arabidopsis* glyceraldehyde-3P-dehydrogenase cytosolic gene (*GapC*). For RT-PCR analysis cDNA synthesis was performed on 1

µg total RNA (DNase treated) using oligo-dT₍₁₅₎ primer. The volume of these cDNA mixtures was increased to 100 µl and for each PCR reaction 5 µl of the cDNA mixtures (equivalent to 0.05 µg RNA) was added to 15 µl PCR mix. Expression of *mac12.6* was detected using the RTL2 primers amplifying a 322 bp fragment from cDNA and a 396 bp fragment from genomic DNA. Expression of *GapC* was detected using the GapC primers, which amplify a 530 bp fragment from cDNA and a 1,300 bp fragment from genomic DNA. Semi-quantitative PCR amplification was performed at 20 cycles. The PCR products were detected by DIG-labelled probes after separation on an agarose gel and Southern blotting. Expression of *LEP* was detected by the RTL1 primers, which amplify a 254 bp fragment 3' of the DNA binding domain of *LEP*. The pLEP primers and combinations of these pLEP primers with the primers 35SleaderF and RTL1R identified the 35S transcript. PCR amplification was performed for 35 cycles and the resulting PCR products were separated on an agarose gel. Autoradiograms of the blots and gel images were scanned and processed using the Adobe PhotoShop computer program.

RACE PCR

The SMART RACE cDNA amplification kit (CLONTECH) was used for 5' and 3' RACE PCR with the LEP5'race, LEP3'race and PROM5'race primers for analysis of the *LEP* transcription start and polyadenylation site and the transcription start site of the 35S transcript, respectively. The resulting PCR products were cloned in the pGEM-T-easy vector (Promega) and at least 10 independent clones were sequenced for each analysis.

Anatomical analysis

Plant material was fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 8 hours at room temperature (RT), dehydrated through an ethanol series and embedded in Epon. Sections (1 µm) were stained with Toluidine Blue and mounted in Epon.

SEM analysis

Plants were grown in tissue culture and fixed for 2 days in FAA (50% ethanol, 5% acetic acid and 3.7% formaldehyde). The material was washed in 70% acetone and further processed as described by Schneitz et al. (1997).

In situ hybridisation with DIG-labelled RNA probes

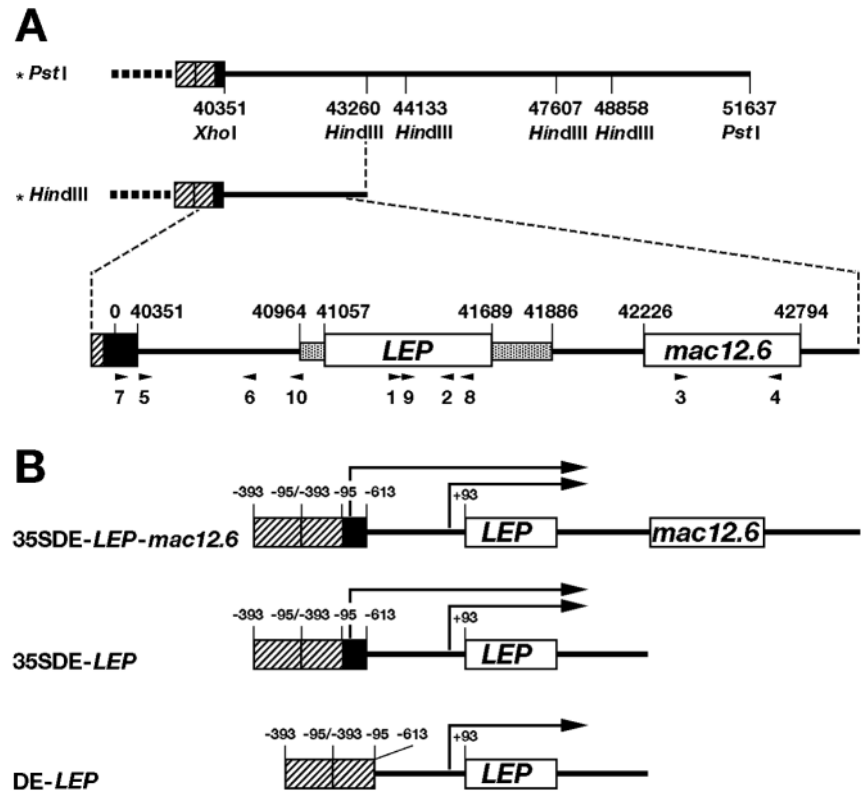
Part of the *LEP* coding region excluding the AP2/EREBP domain was amplified by PCR using the RTL1 primers. The resulting PCR fragment was cloned in the pGEM-T-easy vector (Promega) and DIG-labelled antisense and sense probes were synthesised using the DIG RNA labelling kit (Boehringer) from linearised vectors. Plant material was fixed in FAA for 10-12 hours, dehydrated in a graded ethanol series and embedded in Steedman's wax. Sections (8 µm thick) were cut and mounted on polylysine-coated slides (Sigma), which were baked overnight in a 30°C oven. In situ hybridisation analysis was performed essentially as described by Vielle-Calzada et al. (1999) with minor modifications: Proteinase K (1 µg/ml) was used instead of Pronase E and the postfixation was followed by treatment with acetic anhydride for 10 minutes. The colour reaction was performed using Western Blue (Promega) as substrate (including 7.6 mM levamisole). The slides were stained overnight, washed in TE, mounted in glycerol:TE (1:1) and photographed using Nomarski optics.

RESULTS

Altered development of the *let* mutant

In a screen for leaf developmental mutants we isolated a transgenic line exhibiting a dominant leaf phenotype. Because of its conspicuous leafy petiole phenotype, which consisted of the formation of leaves without a petiole (Fig. 2A-D), this mutant was called *lettuce* (*let*). The total width and length of

Fig. 1. Schematic representation of the T-DNA insert with flanking plant DNA isolated from *let* and the derived T-DNA constructs. (A) Using *Hind*III and *Pst*I, 2.9 kb and 11.3 kb of plant DNA flanking the T-DNA insert was isolated via plasmid rescue. The *Pst*I fragment was subcloned into 5 smaller fragments to generate probes for northern blot analysis using the indicated *Hind*III and *Xho*I sites. Part of the 2.9 kb *Hind*III fragment directly flanking the T-DNA insert is shown in detail. On this fragment two genes are located: *LEP* (41057–41689) and *mac12.6* (42226–42794). *LEP* contains a 93 bp 5'UTR (40964–41056) and a 197 bp 3'UTR (41690–41886). Numbers indicate the location on the BAC clone MAC12. The numbered arrowheads indicate the position of the primers used in this study, as follows. (1,2): RTL1F and RTL1R, (3,4): RTL2F and RTL2R, (5,6,7): pLEPF, pLEPR and 35SleaderF and (8,9,10): LEP5'race, LEP3'race and prom5'race. Stippled box: 5'UTR and 3'UTR region of *LEP*. Broken line: pUC9 sequences present in the activator T-DNA used for plasmid rescue. (B) The T-DNA constructs derived from the 2.9 kb *Hind*III fragment directly flanking the RB (shown in A). This *Hind*III fragment (35SDE-*LEP*-*mac12.6*) was used to generate the 35SDE-*LEP* construct upon removal of the *mac12.6* coding region using the *Acc*I restriction site at position 42174 just upstream of the *mac12.6* coding region. DE-*LEP* was obtained by omitting the 35S minimal promoter region and AMV leader sequence (black box) from 35SDE-*LEP*. Open boxes: *LEP* and *mac12* coding region. Black box: 35S minimal promoter (–90 to 0) + AMV leader sequence. Hatched box: 35S enhancer region (–393 to –95). Arrows indicate the different *LEP* transcripts, which are generated by the T-DNA constructs.



the mutant leaves were comparable to that of wild type (Fig. 2C,D). The largest rosette leaf on wild-type plants was on average 23 mm long and 9 mm wide with the petiole comprising 29% of total leaf length ($n=29$), while for *let* the dimensions for the largest rosette leaf were 25 mm long and 9 mm wide without petiole ($n=15$). The penetrance of the leafy petiole phenotype was not complete in the first leaf pair (Fig. 2R). In *let* the petiole constituted 25% and 24% of total leaf length for the first and second rosette leaf ($n=15$) respectively, while all subsequently formed leaves lacked a petiole. In wild-type plants, petiole length was 45% and 43% of total leaf length for the first and second rosette leaves ($n=29$) respectively, with petiole length gradually decreasing to 4% of total leaf length for the last rosette leaf formed. Leaf blade anatomy of wild-type and mutants leaves was studied. This showed that the size and shape of the cells in the mutant leaves, including the leafy petioles, were comparable to those in wild-type leaves in both longitudinal and cross sections (Fig. 3). Therefore, the lack of a petiole in the mutant leaves was not caused by altered cell elongation but was the result of the conversion of the proximal petiole into a leaf blade.

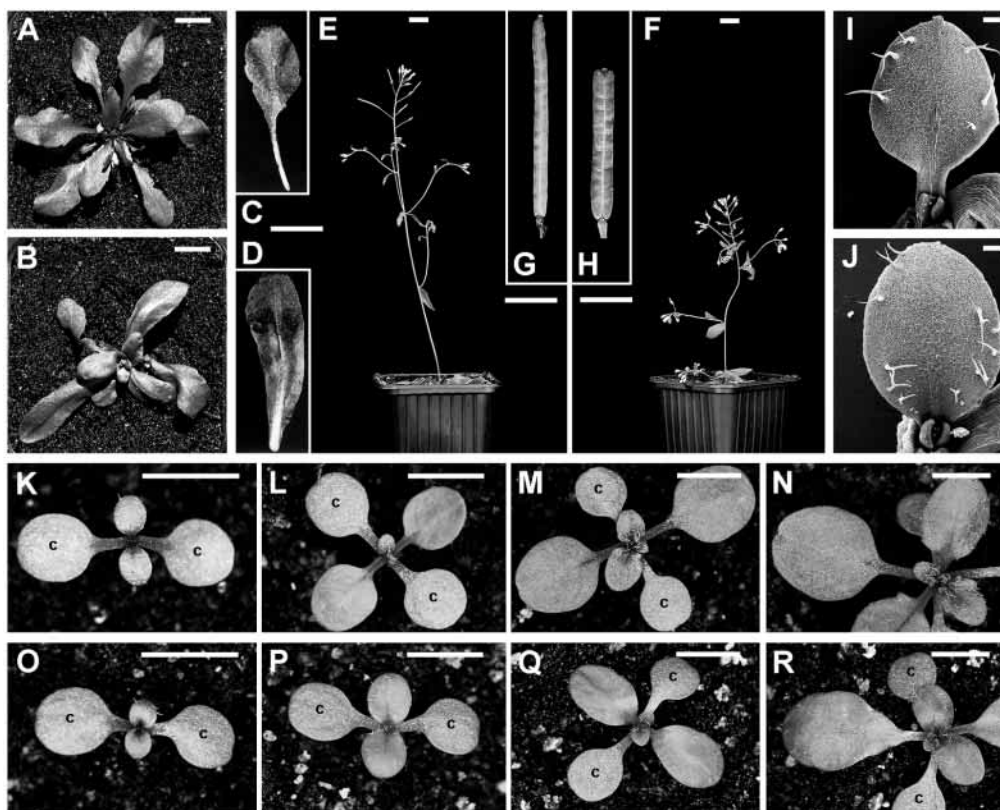
Closer examination of leaf formation using SEM analysis showed that the earliest difference that could be observed between wild type and *let* was in the primary leaves of plants 6 days after germination. At this stage wild-type leaves started to form a petiole (Fig. 2I). No significant petiole was formed on *let* leaves at the same developmental stage, while the leaf blade was larger (Fig. 2J). No difference could be observed in

the morphology of the apex, the leaf primordia or the primary leaves shortly after the outgrowth of the leaf primordia (data not shown). Leaf initiation in *let* (Fig. 2O–R) followed the same phyllotaxis as wild type (Fig. 2K–N). Besides altered leaf development, inflorescence branching of *let* was aberrant (Fig. 2E,F) and the mutant carpels/silques were short and thick (Fig. 2G,H) resembling those formed by the *erecta* mutant (Torii et al., 1996). Cotyledon development was comparable with that of wild-type plants. Although petioles are not formed on cauline leaves, the proximal part of the *let* cauline leaves was wider than those of wild type and resembled mutant rosette leaves in shape. Except for the alteration in carpel/silique shape, the mutant flower organs were identical to wild type (data not shown).

Genetic analysis of the *let* mutant

The T-DNA locus closely linked with the *let* mutant phenotype (see Materials and Methods) and the plant DNA flanking the RB were isolated by plasmid rescue (Fig. 1A). Subfragments of the 11.3 kb flanking plant DNA isolated using *Pst*I (Fig. 1A) were used as probes in northern blot analysis. Only with the 2.9 kb fragment directly flanking the RB of the T-DNA insert as probe was a difference in gene expression detected between wild type and *let* (data not shown). Sequence analysis of the isolated plant DNA and comparison with entries in DNA databases revealed that the isolated DNA showed 100% sequence identity to a region located on the BAC clone MAC12 and, therefore, the T-DNA had inserted on the top (4.6 Mb) of

Fig. 2. The *let* mutant exhibits an altered leaf, inflorescence and silique formation. Development of wild-type (A,C,E,G,I,K-N) and *let* (B,D,F,H,J,O-R) plants. (A-D) 3-week-old seedlings; (A,B) whole plant, (C,D) leaves, (E,F) inflorescence, (G,H) matured siliques. (I,J) SEM images of primary leaves taken from 6-day-old plants. (K-N and O-R) Developmental series of one wild-type and one *let* plant, respectively. Pictures were taken 7 (K,O), 9 (L,P), 11 (M,Q) and 13 (N,R) days after germination. Scale bars, 1 cm (A-F), 100 μ m (I,J) and 0.32 cm (K-R). c: cotyledons.



chromosome V represented by position 40.351 of MAC12 (Sato et al., 1997). Two genes were located on the 2.9 kb fragment directly flanking the RB of the T-DNA insert (Fig. 1A). The T-DNA causing the mutant phenotype inserted 706 bp upstream of the ATG start codon of *mac12.5*, which we call *LEAFY PETIOLE (LEP)* (GenBank AF216581) and 1915 bp upstream of the ATG start codon of *mac12.6*. Southern blot analysis showed that both *LEP* and *mac12.6* are single copy genes (data not shown).

LEP is a member of the AP2/EREBP family

No significant similarity with known genes was previously detected for *LEP* (Sato et al., 1997). However, when we inspected the DNA sequence more carefully, we found that *LEP* encodes a protein that is hydrophilic and contains a region of 57 amino acids which shares a significant similarity with the DNA binding domain of the AP2/EREBP family of plant transcription factors (Weigel, 1995; Riechmann and Meyerowitz, 1998). The protein contains a putative nuclear localisation signal near the N terminus and a proline-rich region between

amino acids 81-105, which might function as an activation domain. Therefore, *LEP* is likely to encode a plant transcription factor. The LEP protein shares the highest similarity with EREBP-3, a member of the tobacco ethylene responsive element binding proteins (Ohme-Takagi and Shinshi, 1995) and as in EREBP-3 the AP2/EREBP domain of LEP is located close to the N-terminus of the protein. The LEP proline-rich region (aa 81-105) showed a high score in

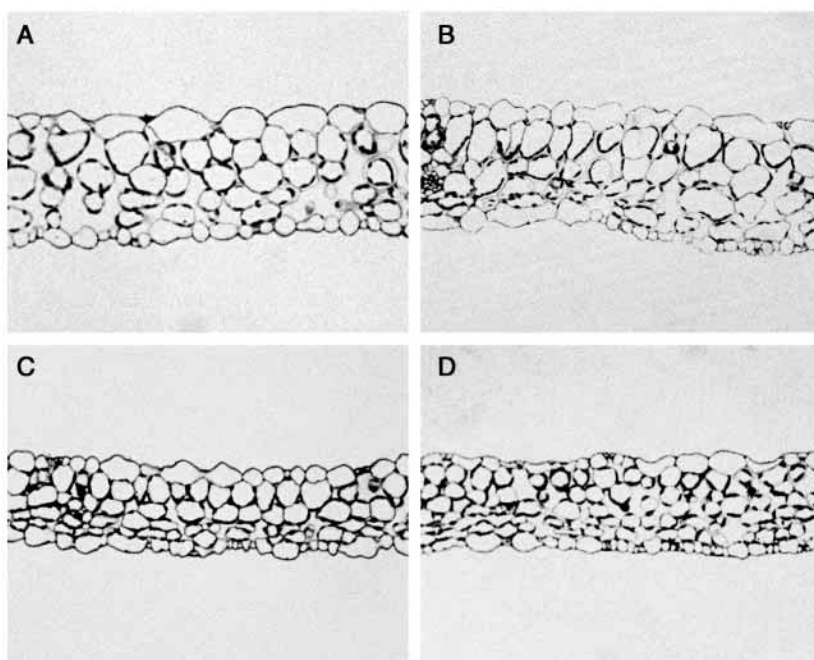


Fig. 3. Anatomy of wild-type and *let* leaf blade. Leaves were isolated from 2-week-old plants grown in soil and sections (A,B, cross; C,D, longitudinal) were made at different positions in the leaf blades. Representative sections are shown for (A,C) wild type, (B,D) *let*. All pictures are shown at the same magnification.

the PESTfind analysis. Such PEST sequences, which are enriched for proline (P), glutamine (E), serine (S) and threonine (T), were found to be responsible for rapid proteolytic degradation (Rogers et al., 1986; Rechsteiner and Rogers, 1996). Examination of a subset of genes belonging to the AP2/EREBP family showed that EREBP1-3, DREB2A, AP2 and RAP2.4 also gave significant scores for the presence of PEST sequences in this PESTfind analysis. On the other hand, no significant scores were obtained for EREBP4, CBF, ANT, TINY, AtEBP and the other RAP and DREB proteins. This suggests that rapid proteolytic degradation could be a feature shared by a subset of genes belonging to the AP2/EREBP family.

Altered expression of *LEP* and *mac12.6* caused by activation tagging

Northern blot analysis showed that *LEP* was very weakly expressed in wild type and could only be detected in young shoots. Wild-type *LEP* expression was most obvious in 1-week-old plantlets and to a lesser extent in 2-week-old shoots (data not shown). *LEP* expression was significantly increased in *let* compared to wild type (Fig. 4A). Two transcripts were detected in *let* with a probe for *LEP*. The smaller transcript migrated the same distance (approximately 900 bp) as the transcript in wild type (Fig. 4A, lane 2) and, therefore, probably originated from transcription initiated on its own promoter, which is likely to be enhanced by the activator tag. The larger transcript was approximately 1800 bp long. Using RACE PCR the position of the putative transcription start and polyadenylation site in *LEP* was determined (Fig. 1A). This showed that *LEP* contains a 93 bp 5'UTR and 197 bp 3'UTR, resulting in a 923 bp transcript without the polyA-tail. Using different primer combinations (35SleaderF, pLEPF, pLEPR and RTL1R; Fig. 1A) the larger transcript was shown to result from transcription initiation from the 35S CaMV promoter, used as activator tag. The start of transcription in the activator tag was confirmed by RACE PCR. The size of this 35S transcript was shown to be 1605 bp without the polyA-tail. None of the hypothetical proteins that could be translated from this 35S transcript, besides the *LEP* protein, was longer than 25 amino acids. Thus, it is unlikely that such a protein would have a function. Wild-type expression of *mac12.6* and even the increased expression levels in *let* could not be convincingly detected in northern blot experiments (data not shown). Therefore, semi-quantitative RT-PCR was performed to study *mac12.6* expression. This showed that *mac12.6* expression was increased approximately 10- to 20-fold in *let* compared to wild type (Fig. 4B).

Activation tagging of *LEP* causes altered leaf, inflorescence and silique development

To study the role of *LEP* and *mac12.6* in the establishment of the altered development of *let*, the 35SDE-*LEP-mac12.6* and 35SDE-*LEP* T-DNA constructs (Fig. 1B) were transformed into wild-type plants. After the transformation of these T-DNA constructs the *let* phenotype (leafy petioles, aberrant inflorescence branching and altered carpel/silique shape) was observed among both sets of primary transformants. The analysis of the progenies derived from the primary transformants showed that most of the transgenic lines exhibited the leafy petiole phenotype, albeit to a varying

degree. Among the 16 35SDE-*LEP-mac12.6* transgenics, 3 lines exhibited a wild-type development, 3 lines a weak, 3 lines an intermediate and 7 lines the strong (comparable to the original *let* mutant) leafy petiole phenotype, while among the 31 35SDE-*LEP* transgenics, 5 lines exhibited a wild-type, 5 lines a weak, 8 lines an intermediate and 13 lines the strong leafy petiole phenotype. *LEP* expression was analysed by northern blot analysis for 12 transgenics (35SDE-*LEP-mac12.6* and 35SDE-*LEP*) exhibiting the leafy petiole phenotype and 3 transgenics displaying a wild-type development. This analysis showed that an increased *LEP* expression could only be detected in the 12 transgenics exhibiting the mutant phenotype. For both the 35SDE-*LEP-mac12.6* and 35SDE-*LEP* transgenics the two transcripts detected in *let* were also observed. No correlation between *LEP* expression levels and strength of the leafy petiole phenotype was observed (Fig. 4D). Because the establishment of the *let* mutant phenotype was observed in both the 35SDE-*LEP-mac12.6* and 35SDE-*LEP* transgenics and only in those transgenics exhibiting an increased *LEP* expression, we conclude that the altered development of *let* was caused by activation tagging of the *LEP* gene.

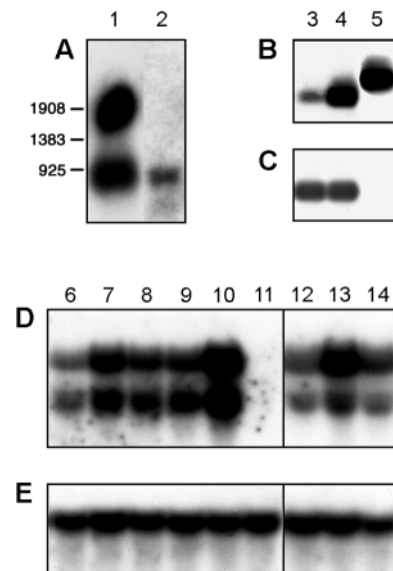
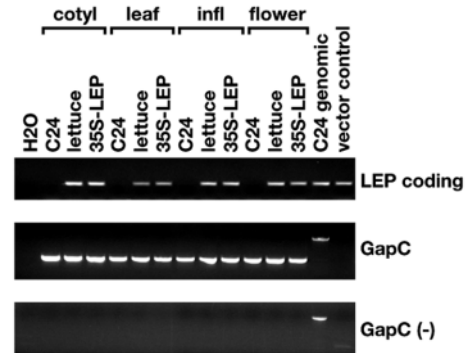


Fig. 4. Two *LEP* transcripts can be detected in *let* and 35SDE-*LEP* overexpressors. (A,D) Northern blot analysis of *LEP* expression. (B,C) semi-quantitative RT-PCR of *mac12.6* and *GapC* expression, respectively. (E) *GapC* loading control. (A) Lane 1: 1 µg poly(A)⁺ RNA from 2-week-old *let* mutant, lane 2: 2 µg poly(A)⁺ RNA from 2-week-old wild type. The filter was hybridised with a probe for *LEP*. The expression shown in lane 1 was obtained after 16 hours and the expression shown in lane 2 after 5 days of exposure. (B,C) Lanes 3 and 4: PCR on cDNA (equivalent to 0.05 µg RNA) from wild type and *let*, respectively. Lane 5: PCR on 0.05 µg genomic DNA from wild type, showing that none of the cDNA samples (lanes 3 and 4) contained a detectable genomic DNA contamination. (D,E) Lanes 6-11: 35SDE-*LEP-mac12.6* transgenics exhibiting a weak (6), intermediate (7 and 8), strong leafy petiole phenotype (9 and 10) and a wild-type development (11). Lanes 12-14: 35SDE-*LEP* transgenics exhibiting a strong (12), intermediate (13) and weak leafy petiole phenotype (14). Total RNA (20 µg) was loaded for lanes 6-14.

Fig. 5. *LEP* is ectopically expressed in most organs of *let* and 35SDE-*LEP* overexpressors. PCR was performed on cDNA (from 0.05 µg RNA) isolated from cotyledons (cotyl), old non-expanding leaves (leaf), inflorescence-stem (infl) and flowers taken from wild-type plants (C24), *let* and a 35SDE-*LEP* transgenic (35S-*LEP*) exhibiting a strong leafy petiole phenotype. As positive controls, PCR was performed on 0.05 µg genomic DNA from wild type or 5 ng vector DNA (containing the 2.9 kb *Hind*III fragment; see Fig. 1A). *LEP* expression (*LEP* coding) was detected using the RTL1 primers and amplification from the individual cDNA samples was verified with GapC primers. PCR using the GapC primers (–) was performed on 0.05 µg RNA as a control for genomic DNA contamination in the different samples.



***LEP* is expressed in leaf primordia and developing leaf blades**

To characterise wild-type *LEP* expression in more detail we studied *LEP* expression in those organs that were developmentally affected in *let* and the 35SDE-*LEP* overexpressors (i.e. the 35SDE-*LEP*-*mac12.6* and 35SDE-*LEP* transgenics displaying the strong leafy petiole phenotype). Because of the low wild-type *LEP* expression levels further analysis of *LEP* expression was performed using RT-PCR analysis (Fig. 5). In wild type no *LEP* expression could be detected in the cotyledons, non-expanding (old) leaves,

inflorescence stems or flowers. Therefore, *LEP* is only expressed in young shoots of wild-type plants. In *let* and the 35SDE-*LEP* overexpressors *LEP* expression was detected in all organs analysed.

Because wild-type *LEP* expression appeared to be restricted to young shoots, the expression of *LEP* was analysed at this developmental stage in more detail with in situ RNA hybridisation (Fig. 6). *LEP* expression patterns at this developmental stage were similar in wild type, *let* and 35SDE-*LEP* overexpressors (Fig. 6B). In wild type, *LEP* was expressed throughout very young leaf primordia just emerging from the

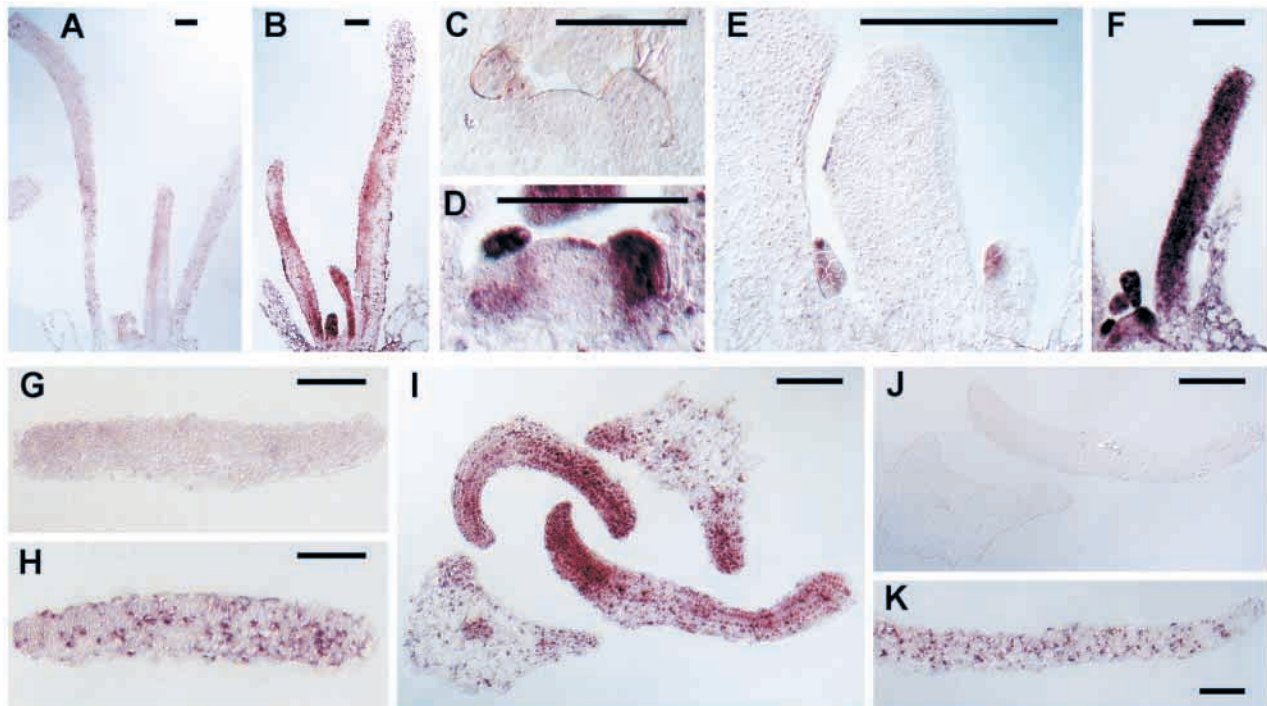


Fig. 6. *LEP* shows similar expression in young shoots of wild type, *let* and 35SDE-*LEP* overexpressors. (A–F) Longitudinal sections through 7-day-old wild-type shoots. (A) No signal was detected with the *LEP* sense probe. (B) Representative staining pattern obtained with the *LEP* antisense probe in wild type, *let* and 35SDE-*LEP* overexpressors. Staining was found throughout the whole leaf primordia with strongest expression in the youngest primordium, gradually decreasing in subsequent older primordia. (C,D) Detail showing the shoot apical meristem region and the emerging leaf primordium hybridised with the *LEP* sense (C) and antisense (D) probe, respectively. (E) Expression in stipules could be detected prior to expression in leaf primordia. (F) Detail of leaf primordium showing strong signal throughout the whole primordium after more prolonged development of the colour reaction than in E. (G–K) Cross sections through 7-day-old wild-type shoots showing leaves at different developmental stages. (G,H) Young developing leaves hybridised with the *LEP* sense (G) and antisense probe (H). (I,J) Young leaves shown at different stages of development hybridised with the *LEP* antisense (I) and sense probe (J). Note the stronger *LEP* expression in the young developing leaf blade compared to the more mature leaf blade and the midrib in I. (K) Older more expanded leaf blade hybridised with the *LEP* antisense probe. Scale bars, 100 µm.

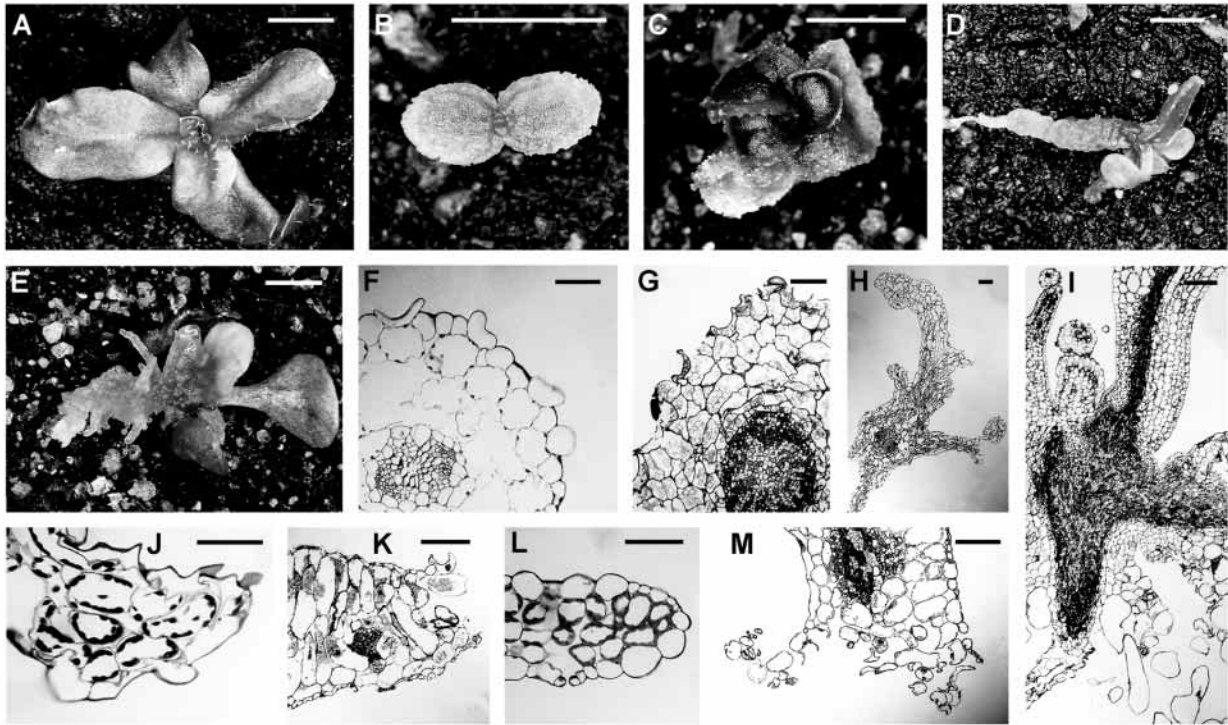


Fig. 7. Development of DE-*LEP* and cDNAoe transgenics. (A) Two-week-old DE-*LEP* transgenic plant exhibiting a strong leafy petiole phenotype. (B-E) Two- to 4-week-old cDNAoe transgenics. (B) cDNAoe-A, (C) cDNAoe-B and (D,E) cDNAoe-C transgenics producing a longer and thicker hypocotyl with callus like appearance (D) and ectopic outgrowths (E). (F-H) Cross section through hypocotyls from wild type (F), and (G) cDNAoe-A and (H) cDNAoe-C transgenics. Note the increase in the number of cell layers in (G) compared to (F) and the ectopic outgrowths in (H). (I) Longitudinal section through a cDNAoe-B shoot. Note the loss of tissue organisation at the base of the hypocotyl and the increase in vascular tissue. (J,K) Cross sections through cotyledons from wild type (J) and cDNAoe-A or cDNAoe-B transgenic (K). (L,M) Cross sections through leaves from wild type (L) and cDNAoe-B transgenic (M). Note the gap at the blade edge in both the cotyledons and leaves from the transgenics (K,M) compared to wild type (J,L). Scale bar, 0.2 mm in (A-E) and 100 μ m in (F-M).

shoot apical meristem (Fig. 6D). Short development of the colour reaction detected *LEP* expression in stipules before expression in leaf primordia could be detected (Fig. 6E), indicating stronger *LEP* expression in stipules compared to leaf primordia. This result was corroborated by analysis of transgenics harbouring a *LEP* promoter (–1670)-GUS fusion construct. In less than 10% of the primary transformants harbouring this promoter-GUS fusion GUS activity was detected in the shoot where it was restricted to the stipules. No GUS activity could be observed in the primordia or young leaves of such lines (data not shown). Longer development of the colour reaction compared to the staining in Fig. 6E revealed staining in all cells constituting the young leaf primordia (Fig. 6F). *LEP* expression could also be detected in young developing leaves where it appeared to be restricted to the leaf blades (Fig. 6H,I). *LEP* expression was strongest in very young leaf blades and became weaker upon further expansion of these blades (Fig. 6L,K). In vacuolated cells containing chloroplasts background staining caused by the chloroplasts interfered with reliable detection of *LEP* expression, thus preventing analysis of leaves at older stages (data not shown). *LEP* expression patterns in *let* and the 35SDE-*LEP* overexpressors were shown to be identical to those in wild type and, therefore, we conclude that *LEP* expression was increased in a tissue-specific way in those organs where wild-type *LEP* expression could be detected.

The 35S transcript initiated in the activator tag is not involved in the establishment of the mutant phenotype

To study the role of the 35S transcript (1800 bp) in the establishment of the mutant phenotype, the DE-*LEP* construct was made (Fig. 1B) in which the 35S minimal promoter was removed in comparison to the 35SDE-*LEP* construct. Primary transformants and their progeny harbouring this DE-*LEP* construct exhibited the leafy petiole phenotype (Fig. 7A), but also the mutant inflorescence and silique/carpel phenotype. Similar to the observation for the 35SDE-*LEP* overexpressors the individual DE-*LEP* lines displayed these phenotypes at different strengths. PCR analysis confirmed that the 35S minimal promoter region was absent in these transgenics (data not shown), while northern blot analysis proved that in the DE-*LEP* lines exhibiting the leafy petiole phenotype only the 900 bp transcript was present (Fig. 8A, lanes 2–4). Because the DE-*LEP* transgenics displayed exactly the same phenotype as *let* and the 35SDE-*LEP* transgenics, we conclude that the 35S transcript does not contribute to the establishment of the mutant phenotype.

Constitutive overexpression of *LEP* causes increased and ectopic cell divisions

To study the effect of constitutive overexpression of *LEP* on plant development, the coding region of *LEP* was amplified by

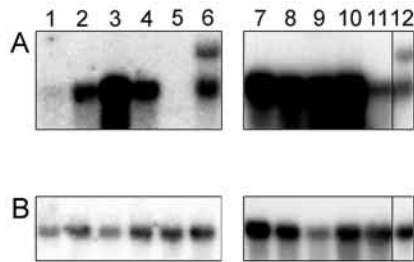


Fig. 8. *LEP* expression in the DE-*LEP* and cDNAoe transgenics. (A) Northern blot analysis of *LEP* expression and (B) *GapC* loading control. Lanes 1-4: four independent primary transformants harbouring the DE-*LEP* construct with (1) wild-type phenotype and (2-4) exhibiting an increase in the strength of the leafy petiole phenotype; lane 5: wild type; lanes 6 and 12: 35SDE-*LEP* transgenic exhibiting a strong leafy petiole phenotype; lanes 7-11: cDNAoe transgenics with (7) old and (8) young cDNAoe-B transgenics, (9) young and (10) old cDNAoe-A transgenics, and (11) cDNAoe-C transgenics. Lane 12 shows a fourfold longer exposure for the *LEP* probe compared to lanes 7-11. In each case 5 µg total RNA (2 µg for lanes 1, 3 and 9) isolated from plants grown in tissue culture was loaded. For the DE-*LEP* transgenics RNA was isolated from single plants, while for the cDNAoe transgenics RNA was isolated from 20 pooled transgenics exhibiting a similar phenotype.

PCR and cloned in a 35S overexpression cassette. The primary transformants harbouring the resulting cDNAoe construct had severe developmental defects (Fig. 7B-M). Three main classes of plants exhibiting distinct altered phenotypes (cDNAoe-A, -B and -C) could be observed. The cDNAoe-A transformants were most severely affected, developed only cotyledons and occasionally very small primary leaves (Fig. 7B). The hypocotyl of such plants was swollen at the base and the edge of the cotyledons was rough rather than smooth (Fig. 7B). The cDNAoe-B plants formed several rosette leaves but they remained small and the plants looked dwarfed (Fig. 7C). In older plants the leaf and cotyledon edge looked rough, and here also, the base of the hypocotyl was swollen. The cDNAoe-C plants formed a minor group and exhibited a longer hypocotyl. A subset of these cDNAoe-C plants had normal or slightly distorted rosette leaf formation. But in all cases the hypocotyl was thicker, with the surface having a callus like appearance (Fig. 7D). In the more severe cases, the hypocotyl had ectopic outgrowths (Fig. 7E). Anatomical analysis of cDNAoe transgenics revealed increased (Fig. 7G) and/or ectopic cell divisions (Fig. 7H). More cell layers were observed in the vascular tissue and cortex of the hypocotyl, and vascular tissue formation was also increased in leaves and cotyledons (Fig. 7I). Leaves produced by cDNAoe-B plants initially showed a wild-type morphology, but upon further development exhibited gaps at the leaf edge resulting in a rough appearance. The formation of the rough cotyledon and leaf edges on cDNAoe-A and -B plants was caused by a loss of connection between ventral and dorsal blade tissue (Fig. 7K,M). Anatomical analysis showed that there is a complete loss of cell organisation in the swollen hypocotyl base (Fig. 7I). Only the vascular tissue seems to remain intact. It proved impossible to obtain seeds from the cDNAoe transgenics since these plants died before bolting when grown in both tissue culture and soil.

Northern blot analysis (Fig. 8A, lanes 7-12) indicated that

LEP was highly overexpressed in the primary transgenics with highest expression in the cDNAoe-A and -B transgenics, which were most severely affected in development. Compared to a 35SDE-*LEP* overexpression line exhibiting the strong leafy petiole phenotype, *LEP* expression was 20- to 50-fold stronger in those cDNAoe transgenics.

DISCUSSION

LEP is a member of the AP2/EREBP family of transcription factors

LEP encodes a putative transcription factor, which shares sequence similarity with the DNA binding domain of members of the AP2/EREBP family of transcription factors (Weigel, 1995; Okamuro et al., 1997; Riechmann and Meyerowitz, 1998). The number of genes constituting this family in *Arabidopsis* has been estimated to be about 125 (Riechmann and Meyerowitz, 1998). The family can be divided into two subfamilies. The EREBP subfamily members contain one DNA-binding domain, while the AP2 subfamily members contain two DNA-binding domains, which may permit these proteins to bind to DNA as a single polypeptide (Moose and Sisco, 1996). In *LEP*, only one such element is present and because of this may require homo/heterodimer formation for DNA binding activity. The functionally characterised proteins containing two DNA-binding domains are known to be involved in the control of development, whereas those harbouring one DNA-binding domain, with the exception of *TINY* (Wilson et al., 1996), are involved in stress-related responses. Apparently, *LEP* is the second protein containing one DNA-binding domain, which is involved in development. It may not be coincidental that *LEP*, like *TINY*, was isolated by activation tagging. *LEP* shares the highest similarity with *EREBP3*, a tobacco gene involved in ethylene-induced pathogen defence. However, activation tagging of *LEP* in *Arabidopsis* caused a leafy petiole phenotype as a result of ectopic leaf blade formation, and thus *LEP* could be involved in leaf blade development.

Altered *LEP* expression caused by activation tagging

In *let* and 35SDE-*LEP* overexpressors, two *LEP* transcripts (1800 and 900 bp, respectively) were identified by northern blot analysis, but in DE-*LEP* plants only the smaller of these two was present. Since all the phenotypes associated with the *let* mutant were also seen in the DE-*LEP* transgenics, the larger 35S transcript apparently does not contribute to the phenotype. Translation of the long transcript, which has a number of small ORFs in front of the *LEP* coding sequence, may not generate *LEP* protein, explaining the lack of contribution of this 35S transcript to the phenotype. The shorter (900 bp) transcript was upregulated in *let* and both the 35SDE-*LEP* and DE-*LEP* transgenics showing an altered development. In situ hybridisation analysis showed that the pattern of *LEP* expression in the young shoot was similar in wild type, *let* and the 35SDE-*LEP* overexpressors. Apparently, the activator tag acts as an enhancer of the *LEP* promoter causing tissue-specific upregulation of *LEP* expression. However, by RT-PCR analysis additional ectopic *LEP* expression was detected in the overexpressors.

Altered leaf development caused by activation tagging of *LEP*

The analysis of wild-type and mutant leaves showed that the leafy petiole phenotype was not caused by a defect in cell expansion. Instead, activation tagging of *LEP* resulted in conversion of the proximal petiole into an ectopic leaf blade; i.e. the leaf blade invaded the petiole region. This suggests that *LEP* might be a positive regulator of leaf blade formation. *LEP* activity could provoke leaf blade formation if *LEP* was required for the establishment of distal domain identity, thereby functioning in proximodistal patterning. Alternatively, *LEP* could be involved in cell division activity in the marginal meristem. The establishment of the leafy petiole phenotype caused by activation tagging of *LEP* could have been brought about by several mechanisms. In situ hybridisation analysis detected wild-type *LEP* expression throughout the whole leaf primordia. Therefore, it is likely that the activity of *LEP* in leaf primordia is regulated at the protein rather than the transcriptional level similar to the proposed regulation of PHAN activity (Waites et al., 1998). *LEP* activity could be suppressed in the proximal domain by either a negative regulator or by increased degradation of the *LEP* protein in this part of the leaf primordia. *LEP* could also interact with other transcription factors encoded by genes exhibiting expression specific for the distal region. Activation tagging of *LEP* might result in higher *LEP* protein levels in the proximal domain of leaf primordia, which might be too high to be completely inactivated or degraded. Alternatively, higher *LEP* protein levels could lead to either homodimer formation or heterodimer formation with partners that *LEP* normally does not interact with. Individual lines harbouring either the 35SDE-*LEP-mac12.6*, 35SDE-*LEP* or DE-*LEP* constructs displayed the leafy petiole phenotype at different strengths rather than an all-or-nothing conversion of petiole to leaf blade suggesting that *LEP* protein levels are related to the degree of leaf blade invasion into the petiole. This can be explained if *LEP* protein activity needs to reach a certain threshold level for stimulation of leaf blade formation.

Wild-type *LEP* expression is restricted to leaf primordia, stipules and developing leaf blades. This expression pattern of *LEP* resembles that of the cyclin *cyc1At* promoter used to study cell division activity during *Arabidopsis* leaf development (Donnelly et al., 1999). That study showed that initially, leaf blade formation is correlated with localised cell division at the primordium margin, while subsequent growth of the tissue layers is established by a more diffuse pattern of cell division in the developing leaf blade. Furthermore, high constitutive *LEP* expression displayed by the cDNAoe transgenics causes increased and ectopic cell divisions. Therefore, *LEP* is likely to act as a positive regulator of cell division activity in both the marginal meristem and during the subsequent establishment of the tissue layers making up the leaf blade.

Altered development caused by ectopic *LEP* expression

Activation tagging of *LEP* not only causes the leafy petiole phenotype but also affects inflorescence and carpel/silique development. High constitutive *LEP* overexpression in the cDNAoe transgenics results in increased and ectopic cell divisions and loss of cell integrity in several organs. It is unclear how ectopic *LEP* expression can result in such diverse alterations

in plant development. Ectopically produced *LEP* might bind to the same target genes as the AP2/EREBP family members, which normally are active in these processes. Consequently, *LEP* would block these target sites for other transcription factors. It is also possible that *LEP* activates these target genes and thus disturbs their normal transcriptional regulation.

Activation tagging

An altered development as observed for the *let* mutant has not previously been described for dicotyledonous plants and this clearly shows that certain (dominant) phenotypes can only be provoked by an increased and/or ectopic expression of tagged genes. Therefore, the use of activator elements in gene tagging studies is complementary to loss-of-function studies, because it provides the opportunity to generate new types of gain-of-function mutants. The *let* mutant could only be isolated because the activation tagging of *LEP* caused a slight alteration in *LEP* expression pattern. Constitutive overexpression by placing the *LEP* coding region under the transcriptional control of the CaMV 35S promoter, a procedure that is normally used to study the effect of overexpression of a gene of interest, resulted in severely deformed plants which were not viable. This indicates that the use of activator tags containing only enhancer elements has the advantage over constitutive promoters that such elements can provoke tissue-specific upregulation of activation-tagged genes, which potentially reveals more about the function of the tagged gene than constitutive overexpression.

Further studies on the role of *LEP* in leaf blade formation, the regulation of *LEP* expression, the possible interaction of *LEP* with other transcription factors and the identification of genes that are regulated by *LEP* is necessary to determine the exact role of *LEP* during plant development. In addition, the *LEP* gene and its homologs from other species may be engineered to increase the yield of crop plants with petiolated leaves.

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